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Report

UCP1 in Brite/Beige Adipose Tissue Mitochondria Is Functionally Thermogenic

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SUMMARY

The phenomenon of white fat "browning," in which certain white adipose tissue depots significantly increase gene expression for the uncoupling protein UCP1 and thus supposedly acquire thermogenic, fat-burning properties, has attracted considerable attention. Because the mRNA increases are from very low initial levels, the metabolic relevance of the change is unclear: is the UCP1 protein thermogenically competent in these brite/beige-fat mitochondria? We found that, in mitochondria isolated from the inguinal "white" adipose depot of cold-acclimated mice, UCP1 protein levels almost reached those in brown-fat mitochondria. The UCP1 was thermogenically functional, in that these mitochondria exhibited UCP1-dependent thermogenesis with lipid or carbohydrate substrates with canonical guanosine diphosphate (GDP) sensitivity and loss of thermogenesis in UCP1 knockout (KO) mice. Obesogenic mouse strains had a lower thermogenic potential than obesity-resistant strains. The thermogenic density (UCP1-dependent oxygen consumption per g tissue) of inguinal white adipose tissue was maximally one-fifth of interscapular brown adipose tissue, and the total quantitative contribution of all inguinal mitochondria was maximally one-third of all interscapular brown-fat mitochondria, indicating that the classical brown adipose tissue depots would still predominate in thermogenesis.

INTRODUCTION

The ability of certain cells in some classical "white" adipose tissue depots to express UCP1 has been recognized for some 30 years (Young et al., 1984) but has recently attracted considerable interest, for several reasons. One is that the degree of induction of UCP1 mRNA in these depots may be very large, up to a 100-fold increase in mRNA levels when the browning process is induced (discussed in Nedergaard and Cannon, 2013). However, because the highest UCP1 mRNA levels in these depots are <20% of those in classical brown depots (Nedergaard and Cannon, 2013; Waldén et al., 2012) and because the UCP1 amount is rate-limiting for thermogenesis as studied in brown-fat mitochondria (Shabalina et al., 2010), it may be questioned whether UCP1 protein will be present in white adipose tissue in sufficient amounts to mediate significant thermogenesis. Indeed, there are only few reports of UCP1 protein (as compared to UCP1 mRNA) in white adipose tissue; it has mainly been detected by immunohistochemistry, and it is found generally in islet-like accumulations of cells. Thus, although immense interest has been vested in the browning phenomenon, indications that the UCP1 is actually associated with significant thermogenic competence are meager if not absent. Therefore, we believe it is important to establish whether the UCP1 expressed in the brite adipose tissues indeed results in thermogenically competent mitochondria with a quantitatively significant thermogenic capacity. We conclude that, at the mitochondrial level, UCP1 protein is present in inguinal white fat in a sufficient amount and with sufficient potential activity to mediate thermogenesis; at the systemic level, the contribution from classical brown-fat UCP1-mediated thermogenesis would nonetheless still predominate.

The "white" adipose tissues and cells that display the ability to express UCP1 are presently mainly referred to as brite (Petrovic et al., 2010) or beige (Ishibashi and Seale, 2010); we use here the brite nomenclature.

RESULTS AND DISCUSSION

Characteristics of Recruited Mitochondria in Inguinal White Adipose Tissue

To examine the thermogenic properties of brite adipose tissue and qualitatively relate these to those in classical brown adipose tissue, we exposed mice to thermoneutrality (30°C; a thermogenically nonrecruited state) or to cold conditions (4°C) for 4 to 5 weeks, i.e., until a new acclimation state had been achieved (a recruited state). These states represent the greatest possible contrast between physiological recruitment states. We then dissected out the inguinal white adipose tissue depot (ingWAT) from these animals; this depot is both the largest brite adipose depot and the one that shows the highest UCP1 mRNA level after cold stimulation (Waldén et al., 2012). For relative evaluation, we also dissected out the interscapular brown adipose tissue depot (IBAT); this depot is the classical brown-fat depot that shows the

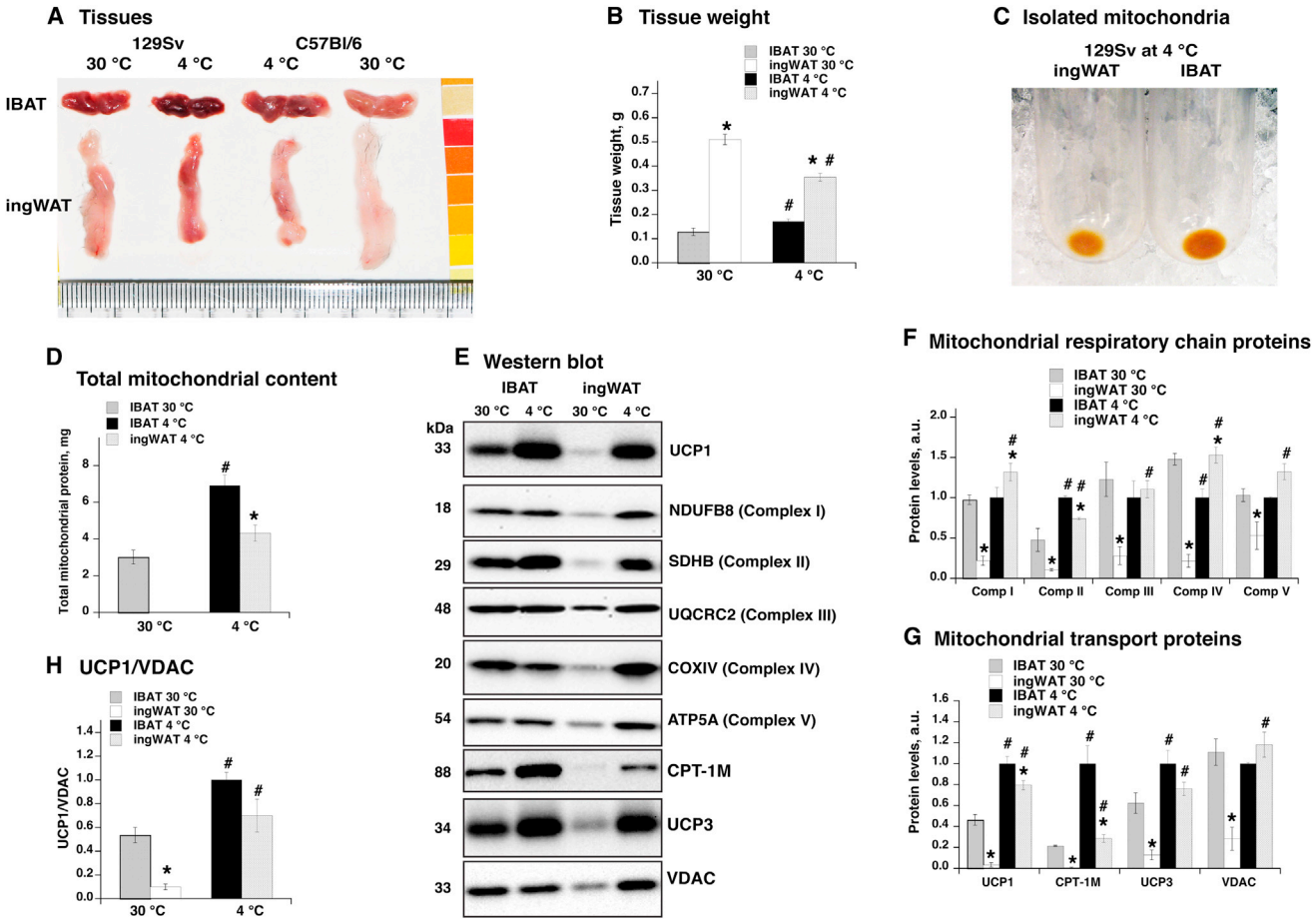

Figure 1. Mitochondria from Brite Adipose Tissue

(A and B) Tissue appearance (A; scale in mm) and tissue wet weight (B; ±SE, n = 7–9) of inguinal white adipose tissue (ingWAT) and interscapular brown adipose tissue (IBAT) of 129Sv and C57Bl/6 mice acclimated to either 30°C or to 4°C.

(C) 129Sv mitochondrial pellets from ingWAT pooled from five male 129Sv mice and from IBAT pooled from three male 129Sv mice acclimated to 4°C during 4 weeks.

(D) Total mitochondrial content per depot corrected for recovery (see Figures S1A–S1C); total mitochondrial content could not reliably be calculated from 30°C ingWAT and is therefore not shown. Preparations were routinely pooled from three to five mice; n = 4–8 independent preparations.

(E) Levels of mitochondrial proteins in purified mitochondria isolated from IBAT and ingWAT of 129Sv mice, acclimated to either 30°C or to 4°C: representative western blots.

(F) Levels of mitochondrial respiratory chain proteins. NDUFB8, subunit of complex I indicated as Comp I, SDHB as Comp II, UQCRC2 as Comp III, COXIV as Comp IV, and ATP5A, α subunit of F1 complex of ATP-synthase, indicated as Comp V. Protein levels in IBAT mitochondria isolated from mice acclimated to 4°C were set to one and the levels in ingWAT mitochondria and in IBAT mitochondria isolated from mice acclimated to 30°C were expressed relative to this value; means ± SE of three to four independent mitochondrial preparations. The asterisk indicates statistically significant difference in protein expression between IBAT and ingWAT mitochondria; Student's unpaired t test ($p < 0.05$); #, statistically significant effect of acclimation temperature; Student's unpaired t test ($p < 0.05$). a.u., arbitrary units.

(G) Levels of mitochondrial transport proteins; statistics as in (F).

(H) Relative UCP1 concentration (calculated from relevant data in G); statistics as in (F) and (G).

highest UCP1 expression (Waldén et al., 2012) and is a major brown adipose tissue depot in mice.

As seen in Figure 1A, cold acclimation led to a visibly much darker/browner appearance of both tissues, in agreement with earlier observations (Cinti, 2005). We examined two mouse strains: the obesity-resistant 129Sv and the obesity-prone C57Bl/6. It is evident that in both tissues and in both conditions, the 129Sv depots were markedly more "brownish" than the C57Bl/6 depots. (These strain differences are further explored below [Figure 4]). For the initial qualitative, mechanistic studies, we used the 129Sv mice. In the text, we will refer to the ingWAT tissue from cold-acclimated mice as brite adipose tissue and correspondingly to brite-fat mitochondria. The weights of the tissues are shown in Figure 1B.

We isolated mitochondria in parallel from the dissected depots. As exemplified in Figure 1C, the mitochondrial pellets from the ingWAT appeared paler and smaller than that from the IBAT. Despite the fact that the ingWAT tissue was much

larger than the IBAT (Figures 1A and 1B), the total mitochondrial content, calculated from experimental yield and recovery (see Figures S1A–S1C), was highest in IBAT (Figure 1D).

If calculated as UCP1 per mitochondrial marker (voltage-dependent anion channel [VDAC]; Figures 1G and 1H), the UCP1 levels were not statistically different between the brite-fat mitochondria and the brown-fat mitochondria. It should be noted that UCP1 was not totally absent in the few mitochondria in the nonrecruited ingWAT (Figure 1H). This implies that the development of brite adipose tissue is not primarily a qualitative shift when seen from a mitochondrial perspective but mainly an augmentation of already existing structures. The brite-fat mitochondria apparently already exist to some extent in the non-recruited tissue; this would be consistent with the (pre)existence of cells in the tissue that are specifically recruited in the cold.

Although all adaptive thermogenesis is mediated by UCP1 (Cannon and Nedergaard, 2010), only within its mitochondrial environment can UCP1 function to dissipate the proton-motive force over the mitochondrial membrane and thus produce heat. We measured the levels of representative subunits of the respiratory complexes I–IV in brite adipose mitochondria (Figure 1E). As seen in the compilation in Figure 1F, for mice in the recruited state, the levels of these subunits per mg mitochondrial protein were remarkably similar between the ingWAT and the IBAT mitochondria; thus, the brite-fat mitochondria were endowed with a high potential capacity in the respiratory chain, allowing for UCP1 function.

Brite-Fat Mitochondria Exhibit Genuine UCP1-Dependent Thermogenesis

A major issue is whether the brite-fat mitochondria are qualitatively similar to brown-fat mitochondria, i.e., display the characteristic oxidation control pattern of genuine brown-fat mitochondria, i.e., a high respiratory activity, fully mediated by UCP1. Alternatively, UCP1, although present, could be inactive or limited by other factors, so that a "normal" mitochondrial respiratory pattern would be observed, i.e., as expected from, e.g., heart mitochondria.

Such a standard heart mitochondrial oxidative control trace is displayed in Figure 2A (mitochondria from cold-acclimated 129Sv mice, isolated in parallel with brite- and brown-fat mitochondria). As seen, heart mitochondria displayed a low initial rate of respiration, even after the addition of substrate (here, palmitoyl-coenzyme A [CoA] or pyruvate). Only after the addition of ADP was the respiratory rate increased. The increased respiration coupled to ADP phosphorylation could be blocked by the ATP synthase inhibitor oligomycin, and the full oxidative capacity on each substrate could be observed after the addition of the artificial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Figure 2A). As seen, practically the full oxidative capacity is utilized during ATP synthesis.

We also examined mitochondria isolated from the epididymal adipose depot, i.e., a depot that has not been reported to show significant browning/britening after cold acclimation (see also Figure S2A). As seen (Figure 2B), the response pattern was very similar to that in heart mitochondria, i.e., there was essentially no respiration prior to ADP addition.

In contrast to these examples of "coupled" mitochondria, we show in Figure 2C (stippled trace) a standard oxidative control trace with mitochondria isolated from the classical IBAT depots of cold-acclimated mice. These mitochondria oxidize substrate at high rates directly when it is added, through the inherent activity of UCP1 (Nedergaard et al., 2001). This high-UCP1-mediated oxidative rate can be fully inhibited experimentally by guanosine diphosphate (GDP) (that is assumed to mimic the high cytosolic ATP levels within the cell). The addition of ADP leads to only a small increase in oxidative rate (see below). A final addition of FCCP reveals the maximal respiratory capacity with this substrate.

Brite-fat mitochondria exhibited a much more brown-fat-like than a heart-like or epididymal-depot-like oxidative control (Figure 2C, full line). The substrate was immediately rapidly oxidized, this high rate was inhibited by GDP, and the subsequent addition of ADP only very modestly induced respiration, with the full respiratory capacity being elicited by FCCP. Data from a series of such experiments are compiled in Figure 2E, juxtaposed to those from brown-fat mitochondria. The maximal levels of respiration were lower in brite- than in brown-fat mitochondria, but basal rates and rates during oxidative phosphorylation were not different between these types of mitochondria; there were thus no qualitative differences. Thus, the UCP1 expression in the brite adipose tissue depots does indeed lead to an inherent potential for nonshivering thermogenesis.

Substrate Selectivity of Brite-Fat Mitochondria

To establish the ability of brite adipose tissue to participate in systemic metabolism, the ability of brite-fat mitochondria to oxidize a series of different substrates, in addition to palmitoyl-CoA described above, was examined.

Pyruvate

In brite-fat mitochondria respiring on pyruvate, the initial rate was UCP1-limited, as the respiratory capacity was greater, as revealed by FCCP addition (Figure 2F). The high-UCP1-mediated capacity for pyruvate oxidation in brite-fat mitochondria implies that the brite adipose tissue mitochondria in situ could readily utilize glucose from the circulation for oxidation; the general inability to observe rodent brite adipose tissue depots with fluorodeoxyglucose positon emission tomography scans is thus not due to an inability of the brite adipose tissue mitochondria to utilize carbohydrate substrate.

Glycerol-3-Phosphate

A low activity of glycerol-3-phosphate dehydrogenase has classically been observed in white versus brown adipose tissue mitochondria (Ohkawa et al., 1969); the difference is due to differences in gene expression (Gong et al., 1998). With glycerol-3-phosphate as substrate, a large difference between thermogenic capacities in brite- and brown-fat mitochondria was observed (Figures 2G and 2H). The lower level of glycerol-3-phosphate dehydrogenase is a direct reflection of a lower gene expression, intermediate between that of epididymal and brown adipose tissue (Figure S2B). Mitochondrial glycerol-3-phosphate dehydrogenase is generally considered to be involved in transferring reducing equivalents from the cytosol to the mitochondrial respiratory system (Houstěk et al., 1975); the low activity may have implications concerning the necessity for reducing equivalent transfer.

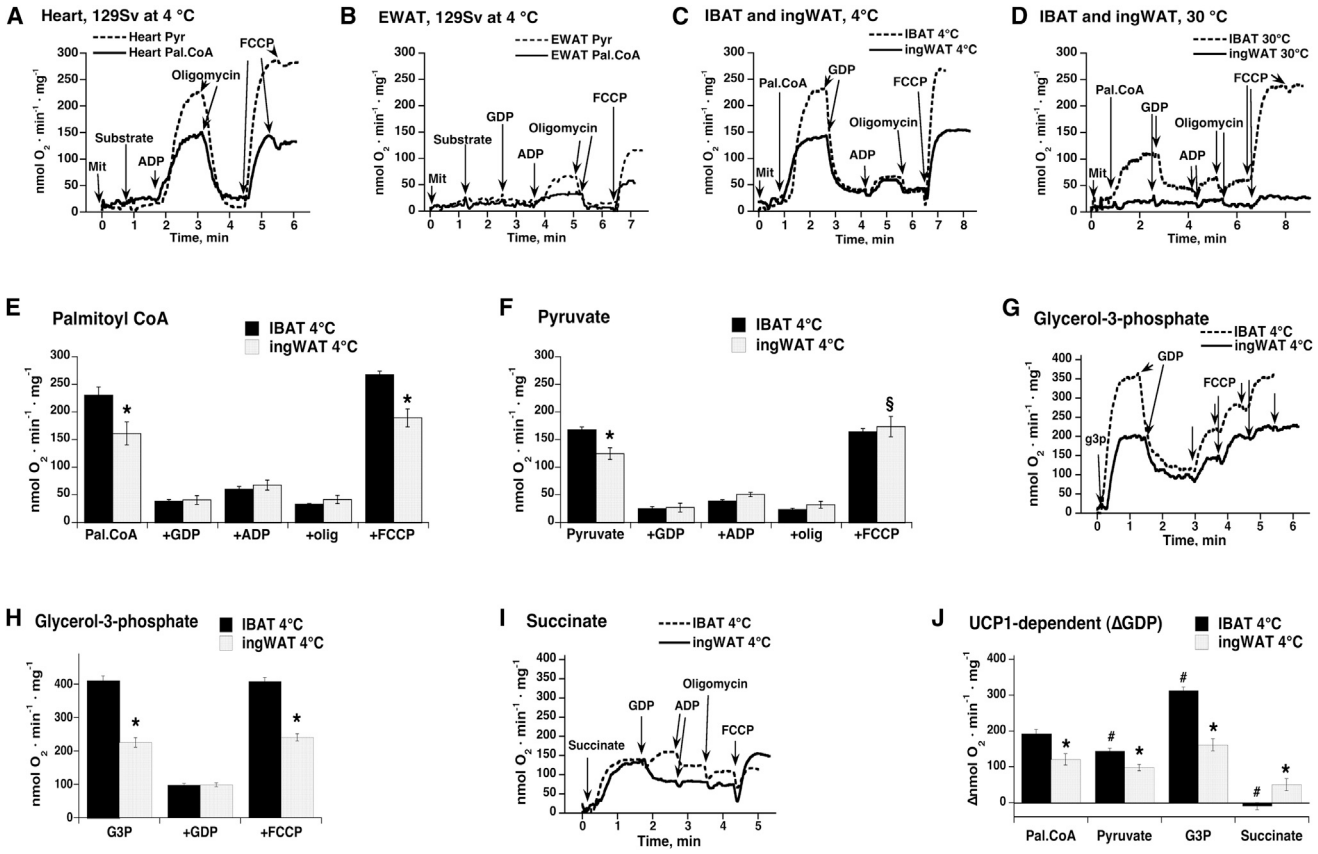

Figure 2. Thermogenic Capacities of ingWAT Mitochondria

All mitochondria (except in D) were isolated from 129Sv mice acclimated to 4°C. Means shown (±SE) are from four to five experiments. For details, see the full experimental procedures.

(A) Representative recording of an oxidative control analysis performed with heart mitochondria. Additions were 0.35 mg mitochondria (*Mit*), 30 μM palmitoyl CoA (*Pal.CoA*, solid line) or 5 mM pyruvate (*Pyr*, stippled line), 450 μM ADP, 2 μg/ml oligomycin, and 1.2–1.4 μM FCCP.

(B) Similar recordings from an oxidative control analysis performed with mitochondria from epididymal adipose tissue; 2 mM GDP added as indicated.

(C) Oxidative control analysis performed with ingWAT (solid line) and IBAT (stippled line) mitochondria; additions as in (A) and (B).

(D) Similar recordings with mitochondria isolated from mice acclimated to 30°C; enlarged version of the ingWAT curve is shown in Figure S2C.

(E and F) Compilations of data from (E) experiments as in (C) and (F) of similar experiments performed in the presence of 5 mM pyruvate; § indicates significant difference between initial and FCCP-induced rate; Student's paired t test $p < 0.01$.

(G) Oxidative control analysis of glycerol-3-phosphate oxidation performed with ingWAT (solid line) and IBAT (stippled line) mitochondria; additions were 0.25 mg mitochondria, 5 mM glycerol-3-phosphate (*g3p*), 2 mM GDP, and final 1.4 μM FCCP.

(H) Compilations of data from experiments as in (G).

(I) Oxidative control analysis of succinate oxidation performed with ingWAT (solid line) and IBAT (stippled line) mitochondria; additions were 0.25 mg mitochondria (*Mit*), 5 mM succinate, 2 mM GDP, 2 μg/ml oligomycin, and final 1.4 μM FCCP.

(J) UCP1-dependent oxygen consumption rates estimated as GDP-inhibitable rates, based on the data behind (E), (F), and (H).

Succinate

For mitochondrial studies, succinate is often chosen as an apparently convenient substrate. In brite-fat mitochondria, succinate addition leads to a fairly high rate of respiration that could be erroneously interpreted as (UCP1-mediated) uncoupled respiration; however, only a small fraction of this succinate-induced respiration could be inhibited by GDP, and the major part of the respiration is therefore not mediated by UCP1 (Figure 2I, full line). It probably occurs in mitochondrial membrane fragments, as suggested for brown-fat mitochondria (Bernson et al., 1979; Shabalina et al., 2010), and is as such an artifact. The small authentic intact mitochondrial succinate respiration in brite-fat mitochondria is probably limited by the capacity of the mitochondrial succinate transporter, as demonstrated for brown-fat mitochondria (Cannon et al., 1984). Thus, analysis of succinate oxidation is noninformative with regard to the question of defining the uncoupled/coupled respiration of mitochondria (and of cells and tissue pieces) in brite adipose tissue.

UCP1-Dependent Thermogenesis

From the above data, UCP1-dependent (thermogenic) oxygen consumption rates were quantified as the GDP-inhibitable rates (Figure 2J). In the brite-fat mitochondria, these rates were approximately similar for fatty acid and carbohydrate oxidation but were somewhat lower than those seen in brown-fat

mitochondria. Thus, per mitochondrion, brite adipose tissue has a somewhat lower thermogenic capacity than brown adipose tissue. The UCP1-dependent oxygen consumption was higher on glycerol-3-phosphate, but this is related to the fact that more oxygen is needed to produce the same amount of ejected protons with substrates that feed electrons directly into coenzyme Q.

Innate Uncoupling as an Adaptive Trait

To examine whether the thermogenic potential observed in brite-fat mitochondria was due to the induction of UCP1 caused by cold acclimation, we compared the oxidative control analysis in brown- and brite-fat mitochondria isolated from mice acclimated to 30°C versus 4°C (Figure 2D versus Figure 2C). In the mitochondria isolated from nonrecruited ingWAT, there was almost no effect of substrate addition (Figure 2D, full line; enlarged in Figure S1C) and there was no effect of GDP addition. The mitochondria were, however, under full oxidative control, because the addition of ADP marginally stimulated respiration, which was oligomycin-sensitive, and FCCP led to a 3-fold increase in the rate of oxygen consumption. Thus, the amount of UCP1 observed in these mitochondria (Figures 1E, 1F, and 1H) was so low that it did not result in measureable thermogenic competence. In brown-fat mitochondria (stippled lines in Figures 2C and 2D), cold acclimation was associated with a doubling of the UCP1-mediated thermogenic capacity (with no change in respiratory capacity), in agreement with the increase in UCP1 density (and with earlier observations Shabalina et al., 2010).

Oxidative Phosphorylation Capacity of ingWAT Mitochondria Is Low

A distinctive characteristic of brown-fat mitochondria versus, e.g., heart mitochondria, is their limited ability to produce ATP through oxidative phosphorylation. This is due to a low content of the ATP synthase itself (Cannon and Vogel, 1977; Houstěk and Drahota, 1977), a result of the feature that all subunits of the ATP synthase are expressed at very high mRNA levels (as are all other OXPHOS proteins)—except for the P1 isoform of subunit c (Andersson et al., 1997; Houstěk et al., 1995; Kramarova et al., 2008). This is considered a very notable feature of rodent brown adipose tissue, and it was therefore of interest to examine whether ATP synthase levels were repressed relative to the respiratory chain also in the brite-fat mitochondria.

This was first examined functionally as part of the oxidative control analysis. As exemplified in Figure 2A, heart mitochondria responded markedly to ADP addition by an increased oxygen consumption rate, underlying the high rate of synthesis of ATP. The response to ADP addition was, as expected, very different in brown-fat mitochondria (Figures 2C and 2D): the response was only 13% of that in heart mitochondria, despite a similar respiratory capacity. In brite-fat mitochondria, the response to ADP was also low, similarly to that of brown-fat mitochondria (Figures 2C, 2E, and 2F). Correspondingly, the level of the alpha subunit of complex V (ATP synthase) was only marginally higher in brite-fat mitochondria than in brown-fat mitochondria (Figures 1E and 1F); in heart mitochondria we found a 10-fold higher level of complex V (alpha subunit; not shown). At the gene expression level, we found that the mRNA levels of the P1 isoform of subunit c (*Atp5g1*) were as low in ingWAT as in IBAT and were significantly lower than in epididymal white adipose tissue (eWAT), liver, or heart (Figure S2D). Thus, the distinctive regulation of ATP synthase in brown-fat mitochondria was duplicated in the brite-fat mitochondria. This type of regulation was, however, not functional in the mitochondria that could be isolated from ingWAT (30°C), indicating that mitochondria of the brite type do not dominate the preparation at that temperature.

Thermogenesis in Brite-Fat Mitochondria Is UCP1-Dependent

Although all the above experiments are indicative of UCP1 being responsible for the thermogenesis seen in brite-fat mitochondria, an unequivocal demonstration can only be made in animals unable to express UCP1. We therefore cold-acclimated UCP1-ablated mice; this is possible through a successive adaptation process (Golozoubova et al., 2001; Ukropec et al., 2006), but the cold-acclimated mice never develop nonshivering thermogenesis (i.e., they continue to shiver; Golozoubova et al., 2001). In the UCP1-ablated mice, both inguinal white adipose tissue and interscapular brown adipose tissue exhibited cold-acclimation-induced changes in appearance (Figure 3A) that were similar to those occurring in wild-type mice (Figure 1A). In the inguinal adipose tissue, the wet weights of the tissue and the mitochondrial yield were unaffected by the ablation of UCP1 (Figure 3B). Unexpectedly, the levels of respiratory chain components (complexes I–IV) in the mitochondria were generally somewhat reduced (Figures 3C–3E) and ATP synthase level somewhat increased, probably due to a somewhat higher level of P1 isoform of subunit c (Atp5g1 in Figure 3F).

In the oxidative control analysis, the response of the brite-fat mitochondria from the cold-acclimated UCP1-ablated mice was similar to that of mitochondria from cold-acclimated wild-type mice (Figures 3G and 3H), except that a low rate of oxygen consumption was maintained after addition of substrate and there was no inhibitory effect of GDP. The response to ADP was similar to that of the wild-type, as was the basal rate after oligomycin, and also the rate after FCCP. The latter was unexpected based on the levels of respiratory chain components (Figure 3D) but may be explainable based on the enhanced expression of carnitine palmitoyl transferase (Figure 3E). We conclude that it is indeed the presence of UCP1 that endows the brite-fat mitochondria with the ability to perform thermogenesis. No compensatory mechanisms for the lost thermogenic ability were observable in the mitochondria lacking UCP1: neither an alternative uncoupled respiration (that would have been visible as an increased rate after oligomycin) nor a significantly increased ability to synthesize ATP to be used in any futile-cycle-related mechanism. These are characteristics also possessed by brown adipose tissue (BAT) mitochondria from UCP1-ablated mice (Matthias et al., 1999).

Innate Differences between Inbred Mouse Strains

As illustrated in Figure 1A, the visible brownness of both the ingWAT and the IBAT depot was markedly lighter in the obesity-prone C57Bl/6 mice than in the obesity-resistant 129Sv mice. The wet weight of the depot was not different between the cold-acclimated 129Sv and C57Bl/6 mice (Figure 4A); however, the mitochondrial yield from ingWAT of C57Bl/6 was significantly

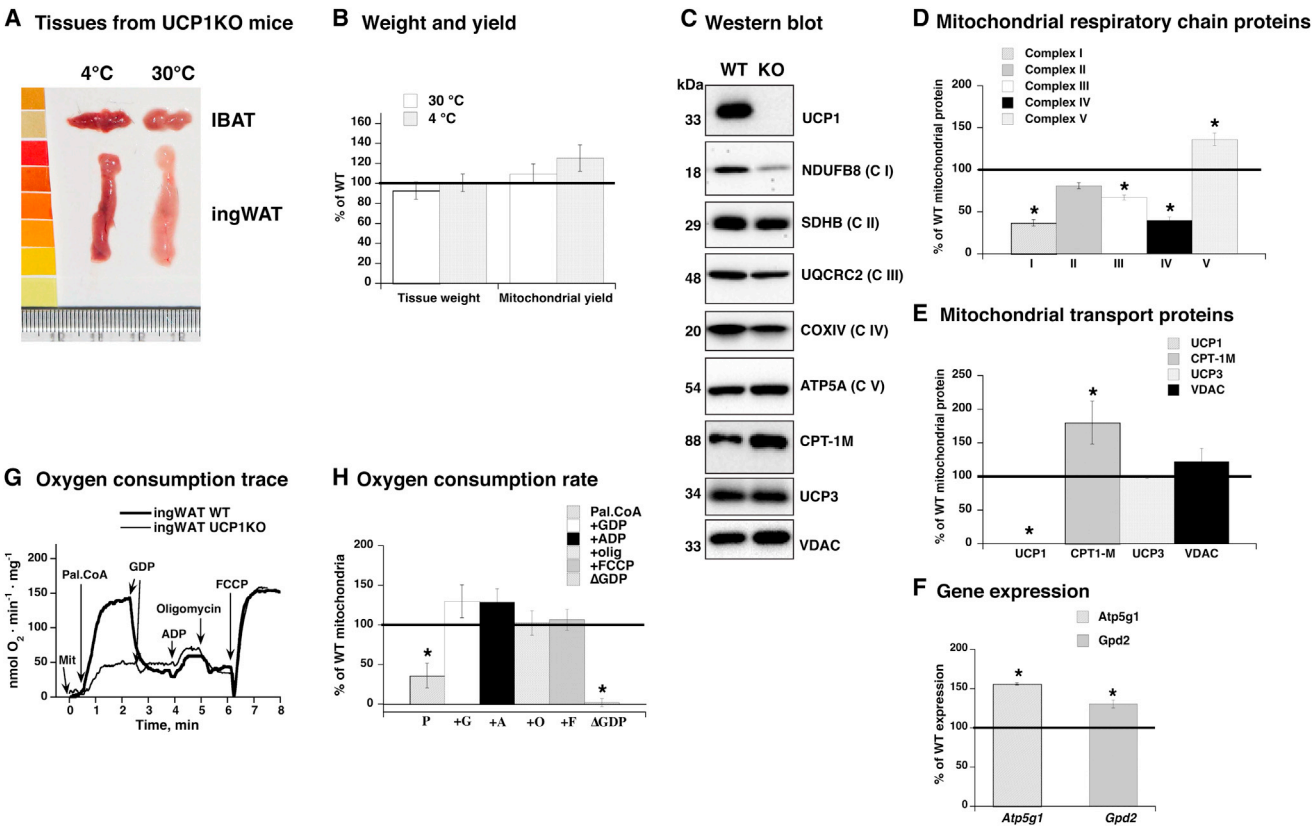

Figure 3. Thermogenic Capacity of ingWAT Mitochondria from Cold-Acclimated UCP1-Ablated Mice

(A) The visual appearance of IBAT and ingWAT isolated from UCP1-ablated mice (UCP1KO) on 129Sv genetic background, acclimated to 4°C.

(B) ingWAT tissue wet weight and mitochondrial yield expressed relative to these parameters in wild-type mice; means from four mice. WT, wild-type.

(C) Representative western blots of mitochondrial proteins in purified mitochondria.

(D and E) Levels of (D) mitochondrial proteins (respiratory chain proteins, subunits as in Figure 1) and (E) mitochondrial transport proteins; the values represent means ± SE of three independent mitochondrial preparations, expressed relative to these parameters in wild-type mice (shown in Figures 1G and 1H).

(F) Expression levels of mitochondrial genes (*Atp5g1* and *Gpd2*). The values represent means ± SE of four independent tissue preparations, expressed relative to these parameters in wild-type mice.

(G) Representative recordings from oxidative control analysis of ingWAT mitochondria form UCP1 knockout (KO) mice (thin line) as compared to wild-type (heavy line); additions were the same as in Figure 2C.

(H) Oxygen consumption rates of ingWAT mitochondria; analyzed as in Figures 1E–1G.

The asterisk indicates statistically significantly different from corresponding protein levels in wild-type mice; Student's unpaired t test ($p < 0.05$).

lower than that observed in 129Sv mice (Figure 4A). To examine whether the C57Bl/6 brite-fat mitochondria were just fewer or also had a thermogenic potential different from that of the 129Sv mitochondria, we analyzed the content of UCP1 and respiratory complexes. As seen (Figure 4B), the brite-fat mitochondria displayed signs of a significantly lower thermogenic potential: lower UCP1, respiratory chain components, and carnitine palmitoyl transferase levels, although VDAC levels were unchanged. These observations were reflected in the oxidative control analysis that showed generally lower thermogenic potential in the C57Bl/6 brite-fat mitochondria, both with palmitoyl-CoA as substrate (Figure 4C) and with glycerol-3-phosphate as substrate (Figure 4D). The calculated UCP1-dependent thermogenic potential was markedly lower in the C57Bl/6 brite-fat mitochondria than in the 129Sv brite-fat mitochondria (Figure 4E).

Thus, in general, a clear tendency to a lower thermogenic capacity of brite-fat mitochondria from C57Bl/6 than from 129Sv mice was seen, which would not be in disagreement with their propensity to develop obesity.

Extrapolation to Systemic Effect

It is possible to obtain some estimate of the systemic contribution of the UCP1-dependent thermogenesis from the brite depots, based on the data shown here, and to compare it with that from the brown depots. The ingWAT depots are larger than the IBAT depots, but it should be realized that the thermogenic density (UCP1-dependent oxygen consumption per g wet weight) of the brite adipose tissue tissues is more than 5-fold lower than that of brown adipose tissue (combining Figures 1B and 2J).

As a first functional approximation of the actual thermogenic capacity of the brite depots, the total thermogenic capacity of ingWAT versus IBAT from cold-acclimated 129Sv and C57Bl/6 mice can be calculated from the UCP1-dependent thermogenic

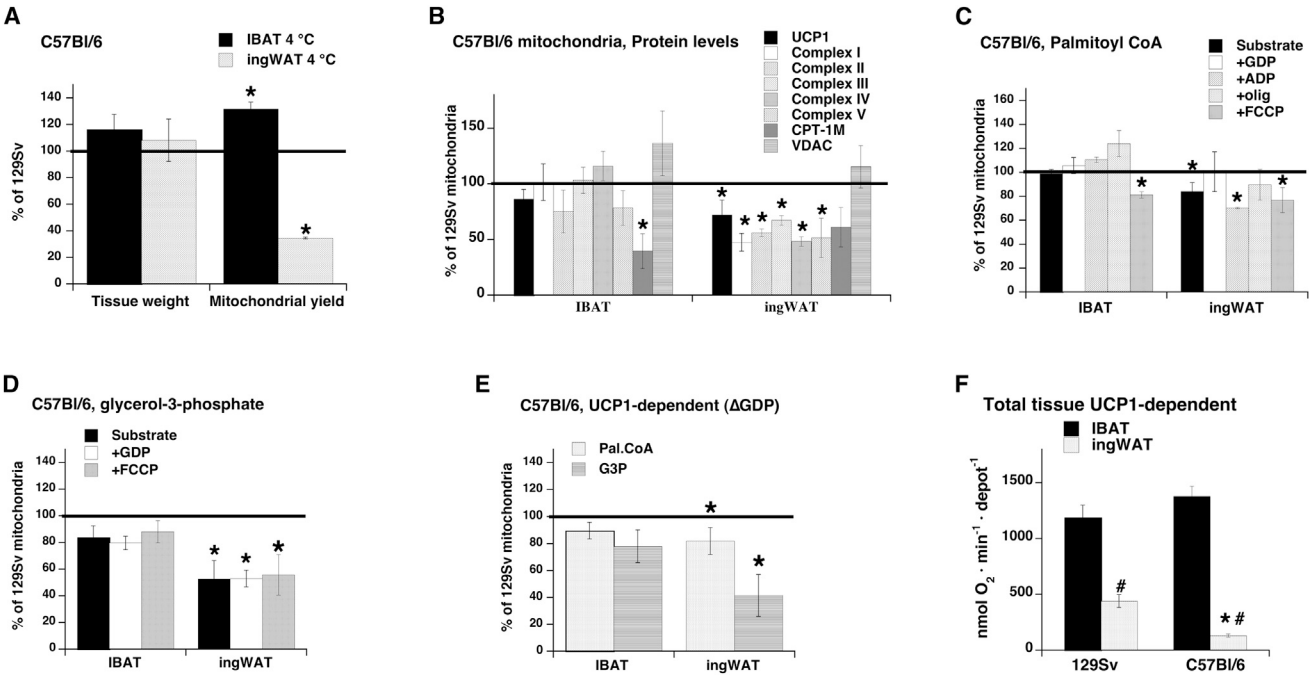

Figure 4. Lower Thermogenic Capacity in Brite-Fat Mitochondria from C57Bl/6 Mice

Relative values for brite-fat mitochondria from C57Bl/6 mice versus those from 129Sv mice; all data are means ± SE from three to five independent C57Bl/6 preparations; * indicates statistically significantly different from corresponding data in 129Sv mice; Student's unpaired t test, $p < 0.05$).

(A) IBAT and ingWAT tissue wet weights and mitochondrial yields.

(B) Levels of mitochondrial membrane proteins.

(C and D) Oxidative control analysis of IBAT and ingWAT mitochondria supported by palmitoyl CoA (C) or glycerol-3 phosphate (D).

(E) UCP1-dependent oxygen consumption rates.

(F) Total recovered UCP1-dependent thermogenic capacity of IBAT and ingWAT from cold-acclimated 129Sv and C57Bl/6 mice, based on the data in Figures 1D, 2J, and 3E; for C57Bl/6 mice, mitochondrial recovery was taken as for 129Sv (Figure S1B).

The asterisk indicates statistically significant difference between 129Sv and C57Bl/6 mice ($p < 0.05$); #, statistically significant difference between ingWAT and IBAT mitochondria ($p < 0.001$).

capacity, estimated as oxygen consumption inhibited by GDP (Figures 2J and 4E), multiplied by total mitochondrial content (Figure 1D), yielding the data shown in Figure 4F. The results in Figure 4F further imply that, in both strains, the thermogenic capacity of the interscapular BAT exceeds that of the inguinal white adipose tissue (WAT), such that the inguinal WAT could represent between 37% (129Sv) and 10% (C57Bl/6) of the thermogenic capacity of the IBAT, under these conditions of strongly induced britening. It would therefore seem that the britening of the white depots would have a positive, although not predominant, influence on the capacity for nonshivering thermogenesis in the situation resulting from stimulation by cold acclimation. It cannot be concluded from these observations whether a thermogenic capacity in specific depots has a distinct physiological role that is different from energy expenditure per se. It may be discussed whether certain other means to bring about britening could result in a higher fractional thermogenic contribution from brite-fat mitochondria, but this has still to be shown. What the present investigation has made clear is that, at the mitochondrial level, UCP1 in brite-fat mitochondria is as thermogenic as it is in brown-fat mitochondria; during recruitment, the brite depots show a much higher relative recruitment, but the classical BAT depots would still predominate in thermogenesis.

EXPERIMENTAL PROCEDURES

Full experimental procedures can be found in the Supplemental Information.

Animals and Mitochondrial Isolation

UCP1-ablated mice were progeny of those described in Enerbäck et al. (1997), backcrossed to the C57Bl/6 or the 129Sv mouse strains; wild-types were C57Bl/6 and 129Sv mice. The mice were fed ad libitum (R70 Standard Diet, Lactamin), had free access to water, and were kept on a 12:12 hr light:dark cycle. Male mice were divided into age-matched (7 to 8 weeks old) groups, caged singly, and acclimated at 30°C or successively acclimated to cold by first placing them at 18°C for 2 weeks with the following 4 to 6 weeks at 4°C (Golozoubova et al., 2001).

IBAT, ingWAT, and eWAT depots were pooled from three to five mice. Mitochondria were isolated in general as described for brown-fat mitochondria in Cannon and Nedergaard (2008). The final mitochondrial pellet was resuspended in 100 mM KCl, 20 mM K^+-Tes (pH 7.2), 1 mM EDTA, and 0.6% fatty-acid-free BSA.

Oxidative Control Analysis

Oxidative control was analyzed in the isolated mitochondria from oxygen consumption rates monitored with a Clark-type oxygen electrode in a sealed chamber at 37°C, as described (Shabalina et al., 2006). The mitochondria (0.35 mg protein/ml or 0.25 mg protein/ml depending on substrate) were incubated in a medium consisting of 125 mM sucrose, 20 mM K^+-Tes (pH 7.2),

2 mM $MgCl_2$, 1 mM EDTA, 4 mM KP_i, and 0.1% fatty-acid-free BSA, with the indicated substrates and inhibitors.

Western Blots and RNA Isolation

Western blots were performed principally as in Shabalina et al. (2006) and Edgar et al. (2009).

For PCR, tissues were dissected and snap-frozen in liquid nitrogen and then homogenized in TriReagent (Sigma-Aldrich), and RNA was extracted with the chloroform-isopropanol extraction method.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.044.

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